

### **REMARKS**

Claims 8-9, 12-14, and 19-23 currently appear in this application. The Office Action of November 5, 2003, has been carefully studied. These claims define novel and unobvious subject matter under Sections 102 and 103 of 35 U.S.C., and therefore should be allowed. Applicants respectfully request favorable reconsideration, entry of the present amendment, and formal allowance of the claims.

### **Rejections under 35 U.S.C. 112**

Claims 15, 16, 25 and 26 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. The Examiner alleges that "kit" renders the claims vague and indefinite.

As the present amendment cancels claims 15, 16 and 24-26, this rejection is now moot.

### **Art Rejections**

Claims 8, 9, 15, 16, and 22-24 are rejected under 35 U.S.C. 103(a) as being unpatentable over The Pharmacological Basis of Therapeutics, hereinafter "Therapeutics" in view of Hukkanen et al. and Moore et al.

This rejection is respectfully traversed. The Examiner states that Therapeutics teaches the method of treating osteoporosis by administering estrogen, thereby inhibiting the

activity of IL-6. However, it is not clear as to whether the inhibition of IL-6 contributes to the treatment of osteoporosis.

Submitted herewith is a copy of Kimble et al., *Journal of Bone and Mineral Research* **12(6)**: 1997, 935-941. This paper discloses that treatment with anti-IL-6 antibody failed to prevent bone loss, and the increase in bone resorption and osteoclastogenesis induced by OVX (see abstract).

Furthermore, Kimble et al. state that while TNF plays a critical causal role in OVX-induced bone loss, IL-6 is not an essential mediator of the effects of estrogen deficiency in bone (see the last paragraph of the DISCUSSION). It is clear from this paper that the inhibition of IL-6 does not directly affect treatment of osteoporosis. In other words, there is no reasonable expectation that administering a selective iNOS inhibitor would successfully treat osteoporosis.

Therapeutics discloses that 17beta-estradiol, a therapeutic agent for osteoporosis, decreases osteoblastic production of IL-6. However, as described above, it is not clear as to whether the inhibition of IL-6 production can treat osteoporosis. Given the Kimmel et al. discovery that treatment with anti-IL-6 failed to prevent bone loss, one skilled in the art would not read Therapeutics as teaching that administering estrogen, which inhibits the activity of

IL-6, would lead one skilled in the art to administer any type of IL-6 inhibitor to treat osteoporosis.

Therefore, the fact that Hukkanen et al. teach that iNOS inhibitors completely inhibit the activity of cytokines such as IL-6 is irrelevant, because, as disclosed in Kimble et al., one skilled in the art would not expect IL-6 inhibitors to treat osteoporosis. Moore et al. add nothing to Therapeutics and Hukkanen et al., as Moore et al. merely disclose that L-N-6-(1-iminoethyl)lysine is a potent iNOS inhibitor.

In view of the above, it is respectfully submitted that the claims are now in condition for allowance, and favorable action thereon is earnestly solicited.

Respectfully submitted,

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## The Functional Block of TNF but Not of IL-6 Prevents Bone Loss in Ovariectomized Mice

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### ABSTRACT

Considerable evidence supports the hypothesis that estrogen prevents bone loss by blocking the bone marrow cell production of pro-osteoclastogenic cytokines. However, controversy remains on the role of candidate factors, such as tumor necrosis factor (TNF) and interleukin-6 (IL-6). To investigate the contribution of these cytokines to the pathogenesis of ovariectomy (OVX)-induced bone loss, OVX mice were treated with either TNF binding protein (TNFbp), an inhibitor of TNF, the anti-(IL-6) antibody (Ab) 20F3, or estrogen for the first 2 weeks after surgery. OVX caused a rapid decrease in trabecular bone volume (TBV) and an increase in *in vivo* bone resorption, as assessed by bone histomorphometry. Treatment with TNFbp completely prevented bone loss and the increase in both osteoclast formation and bone resorption induced by OVX, but had no effects in sham-operated controls. In contrast, treatment with anti-IL-6 antibody failed to prevent bone loss, and the increase in bone resorption and osteoclastogenesis induced by OVX. These data demonstrate that in nongenetically manipulated mice, the estrogen-regulated cytokine that plays a central role in the mechanism by which estrogen deficiency causes bone loss is not IL-6, but rather TNF. (J Bone Miner Res 1997;12:935-941)

### INTRODUCTION

POSTMENOPAUSAL OSTEOPOROSIS is a disorder characterized by a progressive loss of bone tissue which begins after natural or surgical menopause and leads to the occurrence of spontaneous fractures.<sup>(1)</sup> Although the causal role of estrogen deficiency in this condition is well established,<sup>(2-4)</sup> the mechanism by which estrogen prevents bone loss is still controversial. Evidence has now accumulated that suggests that one such mechanism involves the ability of estrogen to regulate the secretion of cytokines that are produced in the bone microenvironment and influence bone remodeling.<sup>(5,6)</sup>

Among these factors are tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and  $\beta$  (TNF- $\beta$ )<sup>(7,8)</sup> and interleukin-6 (IL-6).<sup>(9-11)</sup> TNF promotes bone resorption *in vitro*<sup>(12-14)</sup> and *in vivo*<sup>(15-18)</sup> by activating mature osteoclasts (OCs) indirectly, via a primary effect on osteoblasts,<sup>(17,19)</sup> and by stimulating the proliferation and differentiation of OC precursors.<sup>(20,21)</sup> TNF is also recognized as a powerful inhibitor of bone formation<sup>(14,22)</sup> and as a potent inducer of other pro-oste-

oclastogenic cytokines such as IL-1, macrophage colony-stimulating factor (M-CSF), granulocyte/macrophage colony-stimulating factor (GM-CSF), and IL-6.<sup>(9,23,24)</sup> When bound to the 80 kD subunit of the IL-6 receptor, IL-6 stimulates the early stages of osteoclastogenesis in human and murine cultures,<sup>(25,26)</sup> presumably by forming a complex with gp130 expressed on either stromal cells or osteoblasts.<sup>(27,28)</sup> Consequently, IL-6 increases bone resorption in systems rich in osteoclast precursor, such as the mouse fetal metacarpal assay,<sup>(29)</sup> whereas it has less potent effects in organ cultures where more mature cells predominate, such as murine fetal radii.<sup>(29)</sup>

A causal role for these cytokines in postmenopausal osteoporosis was first suggested by studies from us and others demonstrating that the monocytic production of IL-1 and TNF increases after natural and surgical menopause and decreases with estrogen replacement,<sup>(30-32)</sup> and by the report that the TNF-induced stromal cell production of IL-6 decreases with *in vitro* estrogen treatment<sup>(9)</sup> and increases with estrogen withdrawal.<sup>(11)</sup> The existence of a relationship between cytokines and postmenopausal bone loss was

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further suggested by the demonstration that the bone resorption activity released into the culture medium by peripheral blood monocytes increases after menopause and is blocked by neutralization of IL-1 and TNF<sup>(35)</sup> and by the finding that estrogen replacement decreases the expression of TNF in bone cells from postmenopausal women.<sup>(34)</sup>

However, despite these observations, the relative importance of each cytokine and the mechanism by which they stimulate bone resorption in conditions of estrogen deficiency remains controversial because of the large number of factors involved and their overlapping effects on bone remodeling.<sup>(7,9,35)</sup>

Recently, we demonstrated that TNF, along with IL-1, plays a critical causal role in the rapid bone loss that characterizes the early post-ovariectomy (OVX) period by showing that treatment with TNF binding protein (TNFbp) blocks bone loss in OVX rats and both in vitro OC formation and bone resorption in OVX mice, while it has no effect in estrogen-replete animals.<sup>(36,37)</sup> TNFbp is a divalent inhibitor of TNF, which, by binding specifically and with equal affinity to both TNF- $\alpha$  and TNF- $\beta$ , blocks bone resorption and in vitro osteoclastogenesis.<sup>(37)</sup>

In previous studies, we have also examined the contribution of IL-6 to the increase in bone turnover induced by OVX. This was achieved by treating OVX and control mice with the anti-IL-6 antibody (Ab) 20F3, which is capable of neutralizing the biological effects of endogenous IL-6 for up to 4 weeks.<sup>(10,37)</sup> We found that the functional block of IL-6 with Ab 20F3 does not block the increase in bone resorption induced by OVX. Moreover, Ab20F3 decreased OC formation, not only in cultures of bone marrow from OVX animals, but also in those from estrogen-replete mice.<sup>(37)</sup> These data, which suggest that IL-6 is not an essential mediator of the effects of estrogen in bone, are in apparent contrast with the report that OVX does not induce bone loss in IL-6-deficient knockout mice.<sup>(38)</sup> However, since knockout mice may be characterized not only by the bone remodeling defect induced by OVX but also by modeling abnormalities resulting from altered cytokine production during growth,<sup>(39)</sup> a demonstration that IL-6 deficiency prevents OVX-induced bone loss in a bona fide model of postmenopausal osteoporosis is still lacking.

To investigate the contribution of TNF and IL-6 to OVX-induced bone loss in nongenetically manipulated mice, we assessed the effects of treatment with TNFbp and anti-IL-6 Ab on bone volume and bone resorption in OVX and sham-operated mice. We report that the functional block of TNF prevents OVX-induced bone loss and decreases bone resorption in a manner similar to estrogen, whereas inhibition of IL-6 has no bone-sparing effects.

## MATERIALS AND METHODS

### Study protocol

C3H/Hen mice (Harlan, Indianapolis, IN, U.S.A.) at 5 weeks of age were OVX or sham-operated by the dorsal approach under general anesthesia. Based on the results of preliminary dose response studies (not shown), OVX mice were treated with either 17 $\beta$  estradiol (0.16  $\mu$ g/day,

the lowest dose that maintains a normal uterine weight), TNFbp (1 mg/kg of bw/day), the antimouse IL-6 Ab 20F3 (1 mg/week), TNFbp vehicle or irrelevant isotype-matched antimouse IgG (Organon Teknica Corp., West Chester, PA, U.S.A.), as previously described.<sup>(37)</sup> Sham-operated mice were treated with each of the above substances, except estrogen. TNFbp was kindly provided by Amgen Inc. (Thousand Oak, CA, U.S.A.). Ab 20F3 was a generous gift of John Kenney (Syntex, Inc., Palo Alto, CA, U.S.A.). All treatments were begun at the time of surgery and continued for 2 weeks. Anti-IL-6 Ab and irrelevant IgG were administered by weekly intraperitoneal (ip) injections with the modalities described by Jilka et al.<sup>(10)</sup> TNFbp and TNFbp vehicle were injected subcutaneously every other day. Estrogen was delivered by slow-releasing subcutaneous pellets (Innovative Research of America, Toledo, OH, U.S.A.) implanted in a nuchal subcutaneous pocket. None of the mice exhibited evidence of infectious disease, impaired growth, immunosuppression, or other side effects caused by the treatments. This protocol was approved by the Animal Care and Use Committee of the Jewish Hospital of St. Louis.

### Bone histomorphometry

Two weeks after surgery, the mice were sacrificed and tibiae collected for histomorphometric analysis. At 10 and 3 days before the mice were killed, they were injected ip with 15 mg/kg of bw oxytetracycline hydrochloride (Pfizer Inc., Brooklyn, NY, U.S.A.). The tibiae were defleshed, cut distally at the fibula synostosis, and fixed in 70% ethanol for 48 h. The bones were dehydrated in a graded series of alcohol, changed to 100% acetone, and infiltrated with methyl methacrylate. Following embedding and polymerization, longitudinal sections (5  $\mu$ m thick) were stained with Goldner-Trichrome. Histomorphometric measurements of the percentage of marrow space occupied by trabecular bone (BV/TV), the OC number per millimeter of trabecular bone surface (N.Oc./mm) and eroded surfaces (ES/BS), and the percentage of trabecular bone covered by resorption surface were obtained in two nonadjacent areas of 0.78 mm<sup>2</sup> each, containing secondary spongiosa, starting 0.50 mm from the proximal growth plate, as previously described.<sup>(36)</sup> All histomorphometric measurements were obtained using a Bioquant Morphometric System (R & M Biometrics, Inc., Nashville, TN, U.S.A.) by an investigator blind with respect to type of surgery and treatment.

### Bone marrow cell cultures and characterization of OC formation

At sacrifice, the bone marrow was flushed from femurs with ice-cold  $\alpha$ -minimal essential media ( $\alpha$ -MEM). The cells were collected, pelleted, resuspended in  $\alpha$ -MEM supplemented with 10% fetal bovine serum (FBS) (GIBCO, Grand Island, NY, U.S.A.), and cultured in multiwell plates (Becton Dickinson Labware, Lincoln Park, NY, U.S.A.) at a density of  $2.5 \times 10^6$ /cm<sup>2</sup> for 7 days in presence of 10 nM 1,25-dihydroxyvitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>) as previously described.<sup>(37)</sup> At the end of the culture period, the cells were

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fixed and stained for tartrate-resistant acid phosphatase (TRAP). Expression of calcitonin receptors was also assessed by autoradiography using [ $^{125}$ I] salmon calcitonin (Peninsula Laboratories Inc., Belmont, CA, U.S.A.), as previously described.<sup>(37)</sup> The number of OCs produced by the bone marrow cultures was assessed by counting the number of TRAP-positive multinucleated cells expressing the calcitonin receptor.

*Assessment of serum neutralization activity*

The presence of active TNFbp and anti-IL-6 Ab in the 2 week serum of TNFbp- and 20F3 Ab-treated mice was assessed as previously described<sup>(37)</sup> by measuring the ability of the sera to inhibit the binding of rTNF- $\alpha$  and rIL-6 to immobilized anti-TNF and anti-IL-6 antibodies, respectively. Briefly, sera (20  $\mu$ l) from OVX mice treated with either TNFbp, Ab 20F3, or control substances, and TNFbp (100 pg/ml) or Ab 20F3 (100 ng/ml) were serially diluted (1:2) and incubated with rTNF- $\alpha$  (1 ng/ml) or rIL-6 (0.6 ng/ml) for 3 h at 4°C (total volume 200  $\mu$ l). In parallel, microtiter plate wells were coated with antimurine TNF- $\alpha$  or antimurine IL-6 capture antibodies (Pharmingen, San Diego, CA, U.S.A.). Uncoupled binding sites in the wells were blocked with 3.0% bovine serum albumin. After washing, the sera previously incubated with the rTNF- $\alpha$  or rIL-6 were transferred to the wells and incubated in triplicate overnight at 4°C to allow the binding of free cytokines to the immobilized capture Ab. For detection of TNF- $\alpha$  or IL-6 bound to the capture Ab, 10 ng of biotinylated anti-TNF or anti-IL-6 Ab was added to each well for 1 h and washed three times. The plates were then incubated sequentially with peroxidase-conjugated avidin and ABTS substrate (Boehringer Mannheim Biochemicals, Indianapolis, IN, U.S.A.). Color development was assessed at 405 nm with a Dynatek MR 5000 ELISA reader. Results were expressed as percent optical density change by arbitrarily setting the optical density corresponding to 0.6 ng/ml rIL-6 or 1 ng/ml rTNF- $\alpha$  at 100%.

*Statistical analysis*

The effects of surgery and treatment on histomorphometric indices were analyzed by one-way analysis of variance and the Fisher protected least significant difference test.

## RESULTS

*Effects of in vivo treatment with TNFbp and anti-IL-6 Ab on in vitro OC formation*

OVX and sham-operated mice were treated with TNFbp and anti-IL-6 Ab for 2 weeks, beginning at the time of surgery. The efficacy of these treatments was verified by assessing the ability of sera from TNFbp- or anti-IL-6 Ab-treated mice to block the binding of rTNF- $\alpha$  and rIL-6 to the corresponding capture Ab, in a manner similar to TNFbp or fresh Ab 20F3. These competition experiments confirmed the results of an earlier report<sup>(37)</sup> because they

revealed (not shown) that the sera of mice treated with either TNFbp or anti-IL-6 Ab for 2 weeks are able to neutralize the corresponding cytokine.

The ability of TNFbp and anti-IL-6 Ab to block the in vivo biological effects of TNF and IL-6 on bone was further verified by the ability of in vivo TNFbp and anti-IL-6 Ab treatment to block the increase in OC formation in bone marrow cultures induced by OVX. These experiments confirmed our previous findings<sup>(37)</sup> because they demonstrated that bone marrow cultures from OVX mice produced a higher ( $p < 0.05$ ) number of OCs ( $101 \pm 6$  OC/ $10^6$  bone marrow cells) than those from sham-operated mice ( $45 \pm 6$  OC/ $10^6$  bone marrow cells). We also found that the increase in in vitro OC formation induced by OVX was prevented ( $p < 0.05$ ) by in vivo treatment with either TNFbp ( $55 \pm 9$  OC/ $10^6$  bone marrow cells), anti-IL-6 Ab ( $66 \pm 11$  OC/ $10^6$  bone marrow cells), or estrogen ( $51 \pm 3$  OC/ $10^6$  bone marrow cells). Conversely, treatment with either irrelevant Ab ( $98 \pm 12$  OC/ $10^6$  bone marrow cells) or TNFbp vehicle ( $103 \pm 14$  OC/ $10^6$  bone marrow cells) had no effects on OC formation. These data demonstrate that treatments with IL-6 Ab and TNFbp were both effective in blocking the in vivo biological effects on bone of IL-6 and TNF, respectively.

*Effects of TNFbp and anti-IL-6 antibody on bone volume and in vivo bone resorption*

To determine the effects of TNFbp and anti-IL-6 Ab on bone volume and bone resorption, the proximal extremity of the tibia was analyzed by bone histomorphometry. Figure 1A shows that by 2 weeks after surgery, BV/TV was significantly lower in OVX mice treated with TNFbp vehicle than in sham-operated controls. Treatment with TNFbp completely prevented the decrease in BV/TV induced by OVX, as demonstrated by the fact that OVX mice treated with TNFbp had BV/TV values similar to those of sham-operated mice. In contrast, TNFbp had no effects on BV/TV in sham-operated mice.

Figure 1B shows that OVX mice treated with irrelevant Ab sustained a significant BV/TV loss during the 2 weeks of the study and that treatment with anti-IL-6 antibody failed to prevent OVX-induced bone loss. In fact, at the end of the study, OVX mice treated with irrelevant Ab and those treated with 20F3 Ab had similar BV/TV values. Moreover, treatment with anti-IL-6 Ab had no effects on sham-operated mice.

Consistent with the known potent anabolic effects of estrogen in the mouse,<sup>(40,41)</sup> estrogen treatment (Figs. 1A and 1B) not only prevented bone loss but actually resulted in a significant BV/TV increase.

The effects of the functional block of TNF and IL-6 on OC formation in vivo, an index of bone resorption, was determined by counting the number of OCs adhering to trabecular bone surfaces (N.Oc/mm). Figures 2A and 2B show that N.Oc/mm was about 2-fold higher in OVX mice treated with either TNFbp vehicle or irrelevant Ab than in sham-operated mice. This increase in N.Oc/mm was completely prevented by treatment with either estrogen or TNFbp. Although estrogen-treated OVX mice had a slightly lower

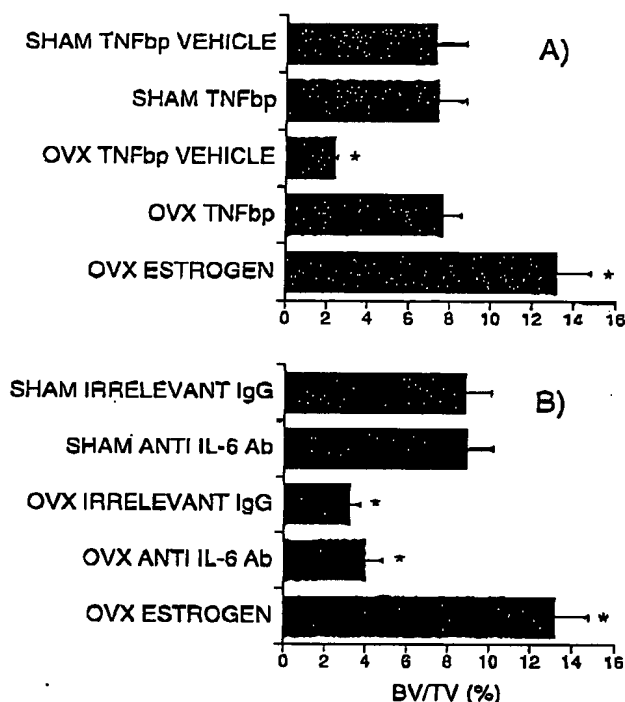


FIG. 1. Effect of in vivo treatment (mean  $\pm$  SEM) with TNFbp and anti-IL-6 Ab on trabecular bone volume (BV/TV).  $n = 10$  mice per group. Mice were OVX or sham-operated and treated with either TNFbp, the anti-IL-6 Ab 20F3, or estrogen for 2 weeks starting at the time of surgery. Percent BV/TV is the percentage of marrow space occupied by trabecular bone. \* $p < 0.05$  compared with sham-operated mice.

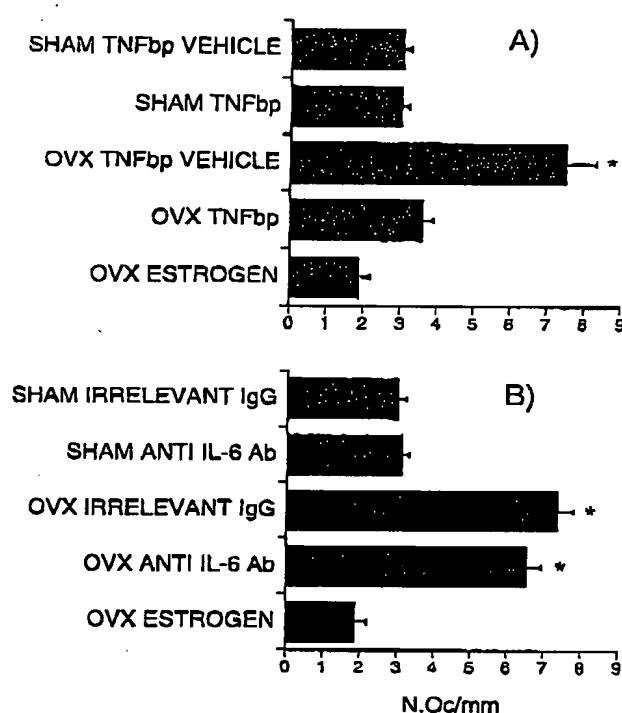


FIG. 2. Effect of in vivo treatment (mean  $\pm$  SEM) with TNFbp and anti-IL-6 Ab on the number of osteoclasts (N.Oc/mm) per millimeter of trabecular bone surfaces.  $n = 10$  mice per group. \* $p < 0.05$  compared with sham-operated mice.

N.Oc/mm than TNFbp-treated mice, this difference was not significant. Conversely, treatment of OVX mice with anti-IL-6 antibody had no effect on N.Oc/mm. Moreover, treatment of sham-operated mice with either TNFbp or anti-IL-6 ab had no effects on OC formation.

In this study, we also measured ES/BS, the percentage of bone covered by resorption surfaces. We found that this index of bone resorption was higher (Figs. 3A and 3B) in OVX mice treated with either TNFbp vehicle or irrelevant Ab than in sham-operated mice. This increase in ES/BS was completely prevented by treatment of OVX mice with either estrogen or TNFbp. Unexpectedly, treatment with IL-6 antibody resulted in a further increase in ES/BS. TNFbp and anti-IL-6 antibody had no effect in ES/BS in sham-operated mice.

We also attempted to analyze the cortical width and bone formation rate. Due to the short duration of the study, we found that at sacrifice cortical width was not significantly lower in OVX than in sham-operated mice. This prevented us from determining if cytokine inhibitors and estrogen prevent cortical bone loss. Similarly, we were unable to determine precisely the dynamic rate of bone formation, due to insufficient labeling of bone surfaces in all groups.

## DISCUSSION

This study demonstrates that neutralization of IL-6 activity does not prevent the bone loss induced by estrogen withdrawal. In contrast, the functional block of TNF prevents bone loss and decreases bone resorption in a manner similar to estrogen.

Inhibition of cytokine activity was obtained by treating OVX mice with either TNFbp or the anti-IL-6 Ab 20F3. TNFbp is an inhibitor of TNF, which at the dose used in this study blocks bone resorption and in vitro osteoclastogenesis for at least 2 weeks in OVX mice.<sup>(37)</sup> The anti-IL-6 Ab 20F3 has been previously used with the same modalities as in this study to neutralize IL-6 in vivo for up to 4 weeks.<sup>(10,37)</sup> In agreement with earlier studies from us and others, we have found that the serum of mice treated with TNFbp and anti-IL-6 Ab were capable of neutralizing rTNF- $\alpha$  and rIL-6 in a manner similar to TNFbp and anti-IL-6 Ab, respectively. We also found that in vivo treatment with both anti-IL-6 Ab and TNFbp blocks the formation of OC in cultures of bone marrow cells from OVX mice, thus demonstrating that the treatment modalities used in this study effectively neutralized endogenous TNF and IL-6 for the duration of the study.

The effects of TNFbp and anti-IL-6 Ab were evaluated in nongenetically manipulated mice to assure that skeletal growth would take place in the presence of physiological

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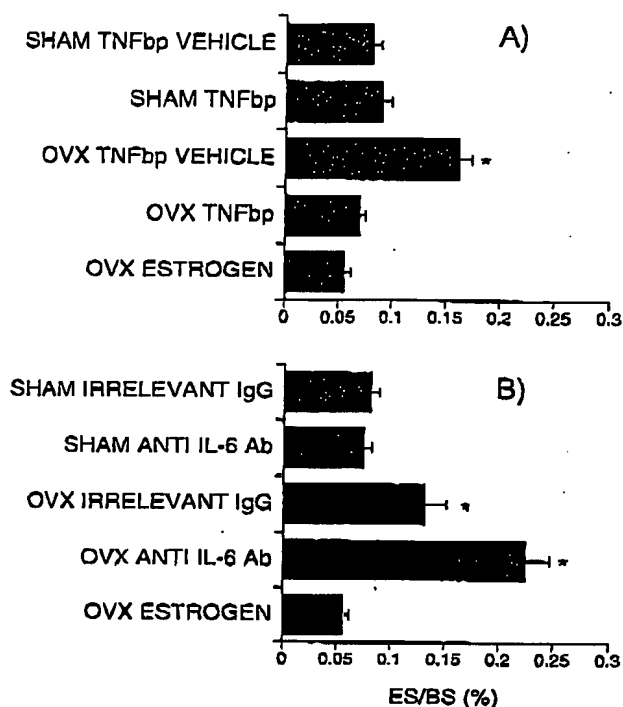


FIG. 3. Effect of in vivo treatment (mean  $\pm$  SEM) with TNFbp and anti-IL-6 Ab on the percentage of bone covered by resorption surfaces (BV/TV).  $n = 10$  mice per group. \*\* $p < 0.05$  compared with sham-operated mice and OVX mice treated with irrelevant IgG.

levels of IL-6 and TNF. This is relevant because postmenopausal osteoporosis is a disease of mature animals. As such, it is characterized by a disorder of bone remodeling which begins in the perimenopausal period, long after the completion of normal bone modeling.<sup>(1)</sup> In contrast, knockout mice lacking the expression of bone-regulating factors are characterized by both modeling and remodeling defects resulting from altered cytokine production during growth. For example, intact IL-6-deficient mice have a decreased bone mass and a greater number of osteoclasts than intact control littermates,<sup>(39)</sup> whereas intact transgenic mice overexpressing IL-6 have an increased number of osteoclasts compared with controls.<sup>(42)</sup> These uncertainties may explain the different results of an earlier investigation conducted using IL-6-deficient mice<sup>(38)</sup> and both more recent studies with the same model and the current study. In fact, studies with IL-6-deficient mice of the third generation which express a stable phenotype revealed that IL-6 deficiency does not protect from the bone loss induced by OVX (R. Balena, personal communication). That IL-6 is not a critical determinant of OVX-induced bone loss is further indicated by the lack of osteopenia and increased OC formation in transgenic mice overexpressing IL-6.<sup>(28,42-44)</sup>

Our findings are not inconsistent with either the ability of estrogen to decrease IL-6 production in murine stromal and osteoblastic cells<sup>(9)</sup> or the ability of anti-IL-6 Ab to

decrease OC formation.<sup>(10)</sup> Since osteoclastogenesis is stimulated not only by IL-6<sup>(10)</sup> but also by other estrogen regulated cytokines,<sup>(20,21)</sup> it could be that in OVX IL-6-deficient mice IL-1 and TNF are sufficient to stimulate bone resorption and promote bone loss. Conversely, since TNF is critical for promoting OC formation,<sup>(9,23,24)</sup> as well as for activating mature OCs,<sup>(17,19)</sup> the functional block of TNF results in a potent inhibition of bone resorption and in a blunted production of "down-stream" cytokines which contribute to the amplification of the OC pool. The ability of TNF to regulate multiple sequential steps critical for bone resorption is, therefore, likely to account for the ability of TNFbp to prevent OVX-induced bone loss.

Several lines of evidence further support the hypothesis that TNF is a critical mediator of the effects of estrogen deficiency in bone. First, estrogen down-regulates the production of this cytokine by bone marrow mononuclear cells,<sup>(37)</sup> osteoblasts,<sup>(8)</sup> and peripheral blood monocytes.<sup>(7,21)</sup> Second, treatment with TNF inhibitors prevents the bone loss and the increase in bone resorption and OC production induced by OVX in rats and in mice.<sup>(36,37,45)</sup> Third, transgenic mice in which the biological effects of TNF are blocked by the overexpression of soluble type I TNF receptors do not lose bone after OVX.<sup>(46)</sup>

Our data demonstrate that treatment of young OVX mice with TNFbp was capable of completely preventing OVX-induced bone loss. In contrast, administration of TNFbp to mature rat decreases, but does not completely block, OVX-induced bone loss.<sup>(36)</sup> This suggests that the specific contribution of TNF to OVX-induced bone loss is either species- or age-dependent.

Although the source and the cellular target of TNF have not been investigated in this study, our working hypothesis is that estrogen deficiency increases the production of TNF from cells of the monocyte/macrophage lineage. Monocytic TNF is, in turn, likely to stimulate osteoclastogenesis by inducing bone marrow stromal cells to secrete a factor capable of promoting the proliferation of hematopoietic OC precursors, such as M-CSF. This hypothesis is supported by recent observations from our laboratory demonstrating that stromal cells from OVX mice have an increased pro-osteoclastogenic activity and produce larger amounts of M-CSF, phenomena which are both prevented by treatment with TNFbp.<sup>(47)</sup>

In summary, these data demonstrate that not all of the pro-osteoclastogenic cytokines known to be regulated by estrogen play a critical causal role in OVX-induced bone loss. While TNF is one such factor, IL-6 is not an essential mediator of the effects of estrogen deficiency in bone.

## ACKNOWLEDGMENTS

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